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The SLC34 family of sodium-dependent phosphate transporters

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ABSTRACT

The SLC34 family of sodium-driven phosphate cotransporters comprises three members: NaPi-IIa (SLC34A1), NaPi-IIb (SLC34A2), and NaPi-IIc (SLC34A3). These transporters mediate the translocation of divalent inorganic phosphate (HPO_4^{2-}) together with two (NaPi-IIc) or three sodium ions (NaPi-IIa and NaPi-IIb), respectively. Consequently, phosphate transport by NaPi-IIa and NaPi-IIb is electrogenic. NaPi-IIa and NaPi-IIc are predominantly expressed in the brush border membrane of the proximal tubule, whereas NaPi-IIb is found in many more organs including the small intestine, lung, liver, and testis. The abundance and activity of these transporters is mostly regulated by changes in their expression at the cell surface and is determined by interactions with proteins involved in scaffolding, trafficking or intracellular signaling. All three transporters are highly regulated by factors including dietary phosphate status, hormones like parathyroid hormone, 1,25- OH_2 vitamin D_3 or FGF23, electrolyte and acid-base status. The physiological relevance of the three members of the SLC34 family is underlined by rare Mendelian disorders causing phosphaturia, hypophosphatemia, or ectopic organ calcifications.

Phosphate homeostasis

Essential role of phosphate

Phosphate is the third most abundant anion in the body and required for a variety of fundamental biologic processes. Inorganic phosphate is essential for bioenergetics (ATP, GTP), metabolic regulation (e.g. in glycolysis or oxidative phosphorylation), intracellular signaling pathways, cell proliferation (as part of the DNA and RNA), and for structures such as bones and membranes [26,25]. Phosphate contributes also to acid-base balance as buffer in blood and in urine. Approximately 85% of total body phosphate is accumulated in bone and teeth, about 14 % is in soft tissues such as skeletal muscle and erythrocytes and only 1% circulates as free phosphate in extracellular fluids. The concentration of intracellular inorganic phosphate may be in the range of 0.7 – 2.5 mM as determined by ^{31}P -NMR and chemical analyses [51]. Extracellular concentrations of inorganic phosphate vary between 0.8 and 1.2 mM in humans. In plasma, phosphate exists in both the monovalent and the divalent form. Based on the pK-value of 6.8, at blood pH of 7.4, 72 % of plasma phosphate is present in the divalent (HPO_4^{2-}) and 28 % is present in the monovalent (H_2PO_4^-) form.

The extracellular concentration of phosphate depends to a large extent on mechanisms that control renal excretion of phosphate. Renal handling of phosphate, and to a lesser extent gastrointestinal absorption of phosphate, is controlled by complex regulatory networks that involve several organs and several endocrine factors [20,38,88,96,98].

Deviations from normal serum phosphate concentrations cause severe clinical disorders. Even slight chronic elevations have been associated with increased rates of death due to cardiovascular complications that are common among patients with chronic kidney disease [131-132,32,115]. On the other hand, prolonged hypophosphatemia, caused by e.g. malabsorption, renal phosphate losses or inherited disorders such as X-linked hypophosphatemia [18], results in symptoms such as osteomalacia, hypercalciuria and bone demineralization [29].

Intestinal phosphate absorption

Organic phosphate ingested in foods is hydrolyzed in the gastrointestinal tract releasing inorganic phosphate. Phosphate absorption along the gastrointestinal tract is mediated by two pathways, a transcellular absorptive component, that involves SLC34 and SLC20 sodium-dependent phosphate cotransporters [54,96,118] and a concentration- or load- dependent absorptive component that may permeate the paracellular route, that, however, is poorly characterized. Together these two processes results in an overall fractional absorption of phosphate that ranges between 65% and 70% of the amount ingested. Notably, segmental distribution of absorption of phosphate varies among different species. In humans and rats most of the ingested phosphate is absorbed in duodenum and jejunum, whereas in mice, most of the phosphate absorption occurs in the ileum [96-97,114]. The role of the colon is uncertain.

Intestinal absorption of phosphate is regulated and adapts to dietary phosphate intake as well as to acid-base status and various hormones such as 1,25-OH₂ vitamin D₃ or glucocorticoids (see below, regulation of NaPi-IIb).

Renal phosphate handling

Phosphate is almost freely filtered by the glomerulus and subsequently reabsorbed along the nephron. The extent of reabsorption depends on dietary intake and a variety of other factors (see below). For the average diet, fractional excretion of phosphate in adults is in the range of 10 – 30 % of the filtered load. There is no evidence for secretion of phosphate. The main site of phosphate reabsorption is the proximal tubule, whether and to which extent more distal segments contribute to phosphate reabsorption has not been fully clarified [21,71-72]. In rodents, the early proximal segments have higher phosphate transport rates and juxtamedullary nephrons are more active than superficial nephrons [21,103-104].

Renal phosphate handling is highly regulated by many factors including dietary phosphate intake, acid-base homeostasis, electrolyte status (e.g. hypokalemia), and a variety of hormones. The renal excretion of phosphate is increased by high dietary intake of phosphate, during acidosis, hypokalemia, by parathyroid hormone (PTH),

1,25-OH₂-vitamin D₃, fibroblast growth factor 23 (FGF23), dopamine, or glucocorticoids. In contrast, increased renal phosphate reabsorption occurs due to low dietary phosphate intake, during alkalosis, or stimulated by insulin, insulin-like growth factor 1(IGF1), 1,25-OH₂-vitamin D₃, and thyroid hormone [103-104,21,17,27].

The SLC34 family of sodium-dependent phosphate transporters

NaPi-IIa (SLC34A1)

The human NaPi-IIa isoform comprises 639 amino acids. Rat and human NaPi-II cDNAs were first isolated by expression cloning [93]. NaPi-IIa is predominantly expressed in kidney and localizes to the apical brush border membrane of the proximal tubule (Fig. 1)[34]. Expression is higher in early convoluted proximal tubules (S1 segments) and in juxtamedullary nephrons but spreads to the late proximal tubule (S2/S3 segments) and to superficial nephrons during phosphate depletion [89]. NaPi-IIa mRNA has also been detected in bone and brain but protein expression has not been confirmed.

NaPi-IIb (SLC34A2)

NaPi-IIb is a protein of 689 amino acids in humans and its mRNA has been detected in many organs including lungs, testis, salivary gland, thyroid gland, small intestine, liver, mammary gland, uterus [68]. In small intestine, NaPi-IIb is expressed at the luminal brush border membrane, in rats and humans in the duodenum and jejunum, whereas in mice mostly in ileum [68,105,114].

NaPi-IIc (SLC34A3)

Human NaPi-IIc comprises 599 amino acids and like NaPi-IIa is mostly expressed in kidney. In kidney, NaPi-IIc is found in the brush border membrane of the early proximal tubule (S1 segment) of juxtamedullary nephrons and is absent from other portions of the proximal nephron (Fig. 1). During phosphate depletion, NaPi-IIc protein is also detected in the early proximal tubule of superficial nephrons

[24,112,121]. NaPi-IIc may also be expressed in bone but its function there has not been elucidated [125].

Structural and functional characteristics of SLC34 transporters

Transport mechanism and kinetic characteristics of SLC34 proteins

The three SLC34 isoforms (NaPi-IIa, b,c) are selective for divalent P_i (HPO_4^{2-}) [8,49] and use the inwardly directed Na^+ electrochemical gradient to catalyse uphill movement of P_i . Although Na^+ and P_i are the preferred substrates, cation replacement studies have established that Li^+ can partly substitute for Na^+ to drive P_i transport, albeit at a significantly lower rate [4]. Transport by NaPi-IIb of the phosphate mimetic arsenate has been demonstrated [138] and given its intestinal localisation this may have relevance for providing a potential route for arsenate accumulation, for example in aquatic species [16]. Transport capacity of SLC34 proteins is also strongly dependent on external pH. As pH defines the monovalent/divalent P_i distribution in the extracellular compartment it may, for example, influence transport rates for NaPi-IIa and NaPi-IIc along the proximal tubule. Protons can also directly modulate the transport kinetics by competing with Na^+ binding and by modifying the apparent rate constants associated with specific kinetic steps in the transport cycle [45].

The SLC34 isoforms display similar apparent substrate affinity constants of $\leq 100 \mu M$ and $\sim 40 mM$ for P_i and Na^+ respectively, at pH 7.4. The transporters probably function close to their maximum rates at least in the initial segment of the renal proximal tubule, given that these apparent affinities are generally well below the typical concentrations found under normal physiological conditions in the intestine and renal proximal tubule. The apparent affinity for P_i is somewhat larger for the mammalian NaPi-IIb compared with the other isoforms ($\sim 10 \mu M$) and this may partially compensate for the reduced availability of divalent P_i in a low pH environment [47,50].

The salient functional feature that distinguishes the three isoforms is whether or not net charge movement accompanies cotransport. NaPi-IIa and NaPi-IIb are electrogenic and translocate one net positive charge per transport cycle. They

mediate transport with a 3:1 $\text{Na}^+:\text{P}_i$ stoichiometry [49]. Importantly, their transport rates are a strong function of membrane potential. In contrast, NaPi-IIc is electroneutral with no net charge translocation and its transport kinetics are insensitive to membrane potential. It mediates transport with a 2:1 stoichiometry [8,122]. The difference in stoichiometries between these isoforms means that the theoretical P_i concentrating capacity is approximately 100-fold higher for NaPi-IIa/b compared with NaPi-IIc, at the cost of a 10-fold greater inward flux of Na^+ ions, together with net charge movement, both of which must be compensated by the cell through the action of the Na^+/K^+ -ATPase.

The transport process for the electrogenic isoforms can be described as a sequence of partial reactions between states that correspond to unique conformations of the protein (Fig. 2). This model is consistent with an alternating access transport mechanism, whereby substrate binding can occur from either the extra- or intra-cellular medium, but not from both simultaneously. The development of this model for the electrogenic NaPi-IIa,b and its extension to include NaPi-IIc has relied on several experimental approaches based on applying the voltage clamp technique to single *Xenopus* oocytes that heterologously express the specific isoform. This technique allows control of a key driving force for transport, membrane potential. Moreover, assays in which both substrate flux (Na^+ , P_i) and net charge translocation are measured simultaneously on the same oocyte have allowed a definitive determination of stoichiometry and specification of the preferred P_i species to be made [49]. Importantly, these assays also confirm the validity of using P_i -induced membrane current as a measure of transport activity. Using steady-state and presteady-state kinetic analysis, electrogenic partial reactions were identified and apparent substrate affinities, turnover rate, pH dependence and the substrate binding order were determined (for review see [47]).

Presteady-state charge relaxations, which result from rapid changes in membrane potential, have proven indispensable to our understanding of the transduction of membrane potential as a transport driving force. They are ubiquitous property of many electrogenic cation driven symport systems (e.g. SGLT1 [63,91], GAT1 [101], EAAT [58]) and provided the first indirect evidence that conformational changes occur as the protein moves from one state to another in response to changes in membrane potential. For NaPi-IIa/b, the transport voltage dependence of arises intrinsically from the proteins themselves that adopt favored orientations

(outward facing or inward facing, Fig. 2), together with Na^+ ion movement to binding sites within the transmembrane electric field (for review see [47]). From measurements of the total charge displacement as a function of membrane potential, estimates of an effective valence per transporter can be obtained, amounting to 0.3-0.4 for the intrinsic carrier and 0.6-0.7 for the carrier with Na^+ bound.

By substituting cysteines at functionally important sites and labeling these covalently with fluorophores, it was established that 2 Na^+ ions bind sequentially and cooperatively before P_i and a 3rd Na^+ binding transition precedes the reorientation of the fully loaded carrier (see Fig. 2) ([142,53]. This conclusion was supported by a detailed cation substitution study in which presteady-state analysis identified the Li^+ interaction site as corresponding to the 1st Na^+ ion binding site [4]. The apparent affinity for Na^+ is determined largely by the first Na^+ interactions, whereas the 3rd Na^+ is a strong determinant of the transport turnover rate. Presteady state kinetic analysis can also be used to estimate the functional expression from the ratio of total mobile charge to effective valence. Moreover, by combining with steady-state electrophysiological measurements, the transport turnover rate can be estimated. For selected electrogenic NaPi-IIa/b this was typically $<100 \text{ s}^{-1}$ and may vary among isoforms and species [50].

The electroneutral NaPi-IIc transport mechanism differs from the electrogenic scheme in that the 1st Na^+ ion to bind to the outward facing empty carrier is not transported (Fig. 2). Thus, for NaPi-IIc, three Na^+ ions bind, but only two are translocated and released to the cytosol [53]. For the electrogenic isoforms, the 1st Na^+ ion is thought to contribute to an uncoupled leak in the absence P_i [3]. Although we propose that Li^+ can also compete with Na^+ for occupancy at the first cation binding site and that in the cotransport mode Li^+ ions can indeed translocate, their contribution to the leak current has not been demonstrated. This “uniport” mode is not detected in NaPi-IIc but when electrogenicity is restored through mutagenesis, the leak mode returns, as evidenced by a change in holding current induced by the inhibitor phosphonoformic acid (PFA), a SLC34 inhibitor [8]. Interestingly, naturally occurring mutations in NaPi-IIc were shown to result in a significant Na^+ -leak [73]. Despite considerable progress in characterizing NaPi-II proteins at the mechanistic level, two areas still deserve attention. First, we are lacking concrete insight into the cytosolic release of substrates, principally because of the experimental limitations of

the intact *Xenopus* oocyte preparation. In one study, NaPi-IIc expressing oocytes were preloaded with tracer substrate and by establishing an outward driving force, reverse transport of P_i was demonstrated in agreement with the kinetic scheme [53]. Recently evidence based on presteady-state analysis of the kinetics of the electroneutral NaPi-IIc, which was modified by mutagenesis to exhibit electrogenic behavior [8], suggests that the last step in the transport cycle is indeed the cytosolic release of one Na^+ ion [110]. This also agrees with earlier studies on the uncoupled Na^+ leak mode [4] in which it was proposed that the leak mode is essentially an uniporter involving the empty carrier and two Na^+ bound states [43]. Further insight will only be obtained using patch clamp or cut-open oocyte techniques to controlling the cytosol composition.

Second, there is a lack of specific high affinity inhibitors for SLC34 proteins. PFA acts as a competitive inhibitor for all isoforms [135,137] and is the compound readily available. However its apparent inhibition constant is relatively large [90] (typically 1 mM (NaPi-IIa), 0.16 mM (NaPi-IIb) and 0.9 mM (NaPi-IIc) [136]) and at high concentrations PFA can induce non-specific leak in the cell membrane (I. C. Forster, unpublished observations). Other commercially synthesized substances with greater efficacies, show mixed competitive and non-competitive behavior (e.g. JTP-59557, Japan Tobacco [99], see also [146]). Having access to specific and high-affinity inhibitors would obviously have considerable potential for control of dietary P_i intake, for which NaPi-IIb is a clinical target.

Structure and structure-function relationships

SLC34 proteins are a unique class of membrane transport proteins and share no obvious homology with the other solute carrier families, even at the bacterial level. At present a 3-D structure of the mammalian SLC34 proteins or their bacterial homologs is lacking, and therefore all structural information has been derived from indirect biophysical and biochemical studies on wild-type and engineered mutations, for review see [47]. NaPi-IIa was shown to be a functional monomer [82], however indirect evidence indicates dimeric or tetrameric structures in the membrane [46,59,56]. The primary sequences of the mammalian isoforms vary from 599 amino acids (NaPi-IIc) to ~640 amino acids (NaPi-IIa and NaPi-IIb). Sequence differences appear mainly in the intracellular C- and N-terminal regions and the large

extracellular loop which contains 2 N-glycosylation sites and a disulfide bridge that links the two halves of the protein (Fig. 3). The proposed secondary topology of the eukaryotic isoforms comprises 8 transmembrane domains (TMDs) and two opposed reentrant loop domains that most likely contain short α -helical motifs. This model incorporates predictions from biochemical and biophysical studies including epitope labeling, cysteine scanning mutagenesis and *in vitro* glycosylation assays (for review see: [44,46,48,139]). The C-terminal region is important for targeting, hormonal regulation and protein-protein interactions. For example, the TRL motif in the C-terminal plays a role as a PDZ binding motif [67,77], and a KR motif located in an intracellular linker region (Fig. 3) is critical for parathyroid hormone (PTH) sensitivity [76]. Of note from the transport function perspective is the signature inverted repeat motif that is conserved among SLC34 isoforms and homologs in all phyla [147]. Cysteine substitution studies (see below) confirm the functional role of these repeats in defining the transport pathway [48,52]. Given that similar motifs are found in the 3-D structures of transporters with identified architecture, e.g. [1,42,120], they most likely contain substrate coordination sites for NaPi-II proteins also. Thus, all SLC34 proteins, including bacterial homologs, are expected to have similar functional core elements comprising TMD's 2-10 (Fig. 3).

Cysteine scanning mutagenesis, in which cysteines are substituted at potentially functionally important sites in the protein and the accessibility by thiol-reactive reagents (methanesulfonates) is determined (e.g. [79]) has been applied extensively to SLC34 proteins (for review see [47-48]) (see Fig. 3). These studies have yielded both structural information as well as mechanistic insights. Substitutions have been made at sites in the predicted re-entrant regions and accessibility studies confirm the reentrant topology [83,87]. Accessibility of some sites is strongly membrane potential and substrate dependent, confirming that the protein conformation is sensitive to these variables [86,142] and cross-linking studies suggest that the two reentrant regions indeed associate [52]. Finally, new insights into the transport mechanism and structure-function relationships have been gained by covalently linking fluorophores to engineered cysteines. The change in fluorescence emission in response to change in membrane potential and external substrate is an indication of specific conformational changes affecting the microenvironment of the fluorophore. In addition to establishing the cation binding

order [142,53,110], they also provide compelling evidence of reciprocal movements of the two halves of the protein during the transport cycle [141].

Finally, insight into the molecular determinants of functional differences between isoforms can be gained by comparing sequences and identifying critical amino acids. For example, the electroneutral NaPi-IIc [122] was used to elucidate the molecular determinants of electrogenicity. Three regions were highlighted that differ significantly in amino acid sequence between electrogenic and electroneutral isoforms. In one of these regions 3 critical amino acids were found by mutagenesis that are conserved in all electrogenic isoforms. Their presence confers electrogenic Na⁺ interactions and transport-dependent charge translocation [8] and recently it was shown that this “re-engineering” of electrogenicity has most likely only restored charge movement to the empty carrier and internal release of one Na⁺ ion [110].

Regulation of SLC34 transporters

Interacting proteins

In vivo and in vitro experiments have shown that NaPi-IIa physically interacts with several proteins in the renal brush border membrane, and that some of these interactions have effects on the expression as well as the regulation of the cotransporter. Most of the known interactions are PDZ (PSD-95, Disc-large, ZO-1)-based. The C-terminal residues of NaPi-IIa (TRL) constitute a PDZ-binding domain that is engaged in association with the PDZ domains of the four members of the NHERF (NHE3 regulatory factor) family [57,56], as well as with Shank2E [100] (Figure 4).

Binding to NHERF1 seems to be critical for the stability of the cotransporter at the brush border membrane, as either preventing the association in cell culture models [66] or the absence of NHERF1 in animal models [128] results in a reduced expression of NaPi-IIa at the cell membrane. Consistently with this reduction, *Nherf1*^{-/-} mice have an increased urinary excretion of phosphate that results in hypophosphatemia [128]. In humans, mutations on NHERF1 have been reported in patients with hypophosphatemia and relatively low TmP/GFR values [78]. NHERF1 has the potential to indirectly connect with the actin cytoskeleton via its association with Ezrin, suggesting that the anchoring of NaPi-IIa to the plasma membrane depends on the formation of a multiprotein complex. Supporting this hypothesis,

Ezrin^{-/-} mice have a reduced expression of NHERF1 and NaPi-IIa at the brush border membrane that leads to urinary loss of phosphate and hypophosphatemia [60]. Therefore, it is not surprising that factors that reduce the expression of NaPi-IIa at the brush border membrane (such as PTH, dopamine or FGF23) induce the dissociation of NaPi-IIa from NHERF1. Treatment of renal proximal tubular cells with PTH leads to phosphorylation of a serine residue (S77) located within the PDZ domain of NHERF1 involved in interaction with NaPi-IIa. NHERF1 phosphorylation at S77 then results in a reduced binding to NaPi-IIa, allowing the retrieval of the cotransporter from the cell surface [35,143]. A similar mechanism has been proposed for dopamine [145] and FGF23 [144]. PTH and dopamine act at least partially via activation of PKC and/or PKA [10,14], and pharmacological activation of either kinase also results in phosphorylation of S77 [143]. Recently, PKA was also shown to phosphorylate Ezrin in vitro, and this modification reduces the interaction of Ezrin with NHERF1 [150]. Phosphorylation of Ezrin may provide an additional molecular mechanism for the destabilization of NaPi-IIa at the cell surface upon hormonal activation. NHERF2 has been suggested to control the intracellular signaling pathway activated upon binding of PTH to its receptor [95]. Thus in the absence of NHERF2, treatment with PTH results in activation of PKA whereas the presence of NHERF2 leads preferentially to activation of phospholipase C (PLC β). This switch is based on the capability of NHERF2 to bind simultaneously to the PTH1R and PLC β , a feature also shared with NHERF1 [95]. Accordingly, mice lacking NHERF1 show impaired activation of PLC by luminal PTH receptors and reduced internalization of NaPi-IIa [30].

The PDZ-binding motif of NaPi-IIa is also responsible for binding to Shank2E [100,36]. Shank2E also interacts with dynamin II, a GTPase involved in the pinching off and endocytosis of clathrin-coated vesicles [107]. Unlike the NHERF proteins, Shank2E is internalized together with NaPi-IIa in response to high phosphate, suggesting that Shank2E controls the intracellular trafficking of the cotransporter.

The interaction of NaPi-IIa with GABARAP (GABA-receptor associated protein) is independent of the PDZ-binding motif [116,56]. GABARAP is known to interact with proteins involved in intracellular trafficking, including tubulin, the N-ethylmaleimide-sensitive factor and clathrin (for review see [102]). GABARAP^{-/-} mice are characterized by reduced urinary excretion of phosphate associated with increased expression of NaPi-IIa; also NHERF1 was found to be upregulated in

mutant animals [116]. The increase in NaPi-IIa does not result in higher levels of phosphate in plasma, probably due to a reduction in the expression of the intestinal NaPi-IIb cotransporter (see below).

Regulation of renal NaPi-IIa and NaPi-IIc

Regulation of SLC34 phosphate transporters has been best defined in the case of NaPi-IIa. In general, regulation occurs mostly on the posttranscriptional level by altering the rate of synthesis and degradation and by changing the amount of NaPi-IIa transporter molecules in the brush border membrane. There is little evidence for a regulation of NaPi-IIa transporters by altering their activity in the brush border membrane through phosphorylation or other related mechanisms.

The biosynthetic pathway and mechanisms governing insertion of NaPi-IIa into the brush border membrane are little known. NaPi-IIa is N-glycosylated and experiments in *Xenopus* oocytes indicated that lack of glycosylation is not altering transport characteristics but decreased membrane expression [62]. Thus, glycosylation may be required for insertion into and/or stability in the plasma membrane.

Retrieval of NaPi-IIa from the brush border membrane occurs rapidly within minutes after injecting PTH into rodents or after increasing dietary phosphate intake [13,24]. NaPi-IIa is internalized via a route involving clathrin-coated pits as well as early and late endosomes and is routed to lysosomes for degradation [13,81,111,134]. There is no evidence for recycling of internalized NaPi-IIa molecules. The pathway of NaPi-IIa internalization is shared with receptor-mediated endocytosis occurring in the same cells of the proximal tubule. The integrity of the machinery involved in receptor-mediated endocytosis is required for NaPi-IIa internalization. Genetic deletion of the major endocytic receptor megalin or its chaperone Receptor-Associated Protein (RAP) alters the structure of the subapical compartment and reduces steady-state as well as PTH provoked endocytosis leading to accumulation of NaPi-IIa in the brush border membrane and enhanced phosphate reabsorption [9,11].

As indicated above, internalization of NaPi-IIa is initialized by phosphorylation of NHERF1 at serine 77 and subsequent dissociation of NaPi-IIa and NHERF1. NaPi-IIa is internalized whereas NHERF1 and other NaPi-IIa associated proteins remain at the apical membrane [35,143]. There is no evidence for phosphorylation of NaPi-IIa by parathyroid hormone. The exact molecular mechanisms that address NaPi-IIa to the lysosome and are required for its routing are presently unknown.

In most cases NaPi-IIa internalization appears to occur without direct modification of the NaPi-IIa transporter in the plasma membrane. However, downregulation by FGF23-klotho may involve also an additional mechanism. FGF23 - klotho may induce direct phosphorylation of NHERF1 and internalization of NaPi-IIa [5]. In addition, klotho alone may proteolytically degrade NaPi-IIa located in the plasma membrane through a beta-glucuronidase-like mechanism. Klotho seems to first modify glycan residues on NaPi-IIa, allowing for cleavage of NaPi-IIa inducing its lysosomal degradation [69].

Regulation of NaPi-IIc abundance and activity is much less well studied. NaPi-IIc removal from the brush border membrane in response to PTH or intake of phosphate-rich diets occurs with much slower kinetics than for NaPi-IIa and requires hours [112,127,126]. In contrast to NaPi-IIa, NaPi-IIc may undergo partial recycling [126].

Regulation by signaling networks and kinases

Activation of parathyroid hormone receptors down-regulate NaPi-IIa abundance at the brush border membrane within minutes through at least two different signaling pathways involving either PKA or PKC. Both pathways may eventually merge in ERK1/2 and lead to the phosphorylation of NHERF1. PTH leads also to downregulation of NaPi-IIc, however, this requires hours and the signals mediating this effect are not known [112].

The signals mediating the down-regulation of NaPi-IIa and NaPi-IIc by FGF-23 are not fully elucidated. FGF23 signals through binding to the FGF1c and FGF4 receptors together with its co-ligand klotho to reduce NaPi-IIa and NaPi-IIc protein

and mRNA abundance [119,75,124]. FGF1c and klotho are expressed in the proximal tubule and with much higher abundance in the distal convoluted tubule. Activation of FGF1c causes phosphorylation of FRS2a and ERK1/2 and acts downstream on Egr1 [85]. Injection of FGF23 into mice increases staining for phosphorylated ERK1/2 in the distal convoluted tubule and it has thus been suggested that the distal convoluted tubule may generate signals acting on the proximal tubule to reduce phosphate reabsorption [37]. Alternatively, FGF23 may directly activate in the proximal tubule a signaling cascade involving sgk1, ERK1/2, and NHERF1 phosphorylation to regulate NaPi-IIa [5].

Also activation of guanylate cyclases and increased intracellular cGMP production reduces NaPi-IIa expression in the brush border membrane. Atrial natriuretic peptide and NO may reduce renal phosphate reabsorption involving cGMP dependent retrieval of NaPi-IIa [12].

Little is known about signals involved in the upregulation of NaPi-IIa expression and stimulated renal phosphate reabsorption. The insulin-like growth factor 1 (IGF1) stimulates NaPi-IIa expression and activity and may mediate some of the age and growth dependent effects on renal phosphate handling. The phosphorylation and activation of tyrosine kinase may be required for the IGF1-dependent effects [74].

Various additional kinases are involved in the regulation of NaPi-IIa function as evident from gene-modified mouse models. However, it is not clear in all instances if this is by directly affecting signaling networks in the proximal tubule or more indirectly by primarily altering the regulation of hormones acting on the kidney. A role for GSK3, Akt2/protein kinase beta 2, Jak2, Osr1, Spak and Sgk3 has been found [108,41,129,109,80].

Regulation of NaPi-IIb

The abundance of NaPi-IIb at the apical membrane of the small intestine is under the control of a number of factors. Early studies indicated that phosphate absorption across the intestinal epithelia decreases during the suckling-weaning transition [23,39,22]. This reduction in the phosphate absorbing capacity coincides with an increase in the plasma levels of corticosterone, a glucocorticoid involved in

intestinal maturation [65]. Arima *et al* found that corticosterone administration results in downregulation of NaPi-IIb at the mRNA and protein level [6], an effect that may underlay the ontogenic regulation of the cotransporter.

As for the renal cotransporters, reduction in dietary intake of phosphate results in increased expression of NaPi-IIb in the intestinal epithelia [61]. This regulation is most probably independent of 1,25-OH₂ vitamin D₃, as it is preserved in VDR^{-/-} as well as 1 α OHase^{-/-} mice [31,123]. There are some discrepancies regarding whether or not the dietary adaptation involves changes on NaPi-IIb mRNA expression, with reports in favor [123,114] and against [61,31] transcriptional regulation. Recently it was shown that NaPi-IIb and NHERF1 are coexpressed in intestinal epithelia and that they interact with each other via a C-terminal PDZ-binding motif of the cotransporter [55]. Furthermore, the dietary upregulation of NaPi-IIb is blunted in NHERF1^{-/-} mice. Improper dietary regulation of the cotransporter has also been described in mice deficient for myosin VI [64]. Myosin VI is a minus-end actin-motor protein involved in a wide range of cellular functions including clathrin-mediated endocytosis. Hegan *et al* reported that feeding a low Pi diet results in high and similar levels of NaPi-IIb in wild type and myosin VI^{-/-} mice. However, upon administration of high phosphate the downregulation of the cotransporter at the cell surface is smaller in myosin VI^{-/-} than wild type mice [64]. These findings suggest that myosin VI is required for the membrane retrieval of the cotransporter. In addition to NHERF and myosin VI deficient mice, the expression of NaPi-IIb is also altered in GABARAP^{-/-} mice. As indicated above, NaPi-IIb expression is reduced in these mice, most probably as a compensatory mechanism for the increased expression of the renal NaPi-IIa ([117,116].

Although not required for the dietary adaptation, 1,25-OH₂ vitamin D₃ directly upregulates the expression of NaPi-IIb [61]. Moreover, the abundance of NaPi-IIb (protein and mRNA) is reduced in the intestinal epithelia of VDR^{-/-} mice [123,31]. Experiments in rats indicated that the effect of 1,25-OH₂ vitamin D₃ on NaPi-IIb expression involves changes in mRNA transcription in young but not in adult animals [148]. This last report also shows that the activity of the human NaPi-IIb promoter increases in response to 1,25-OH₂ vitamin D₃. The levels of 1,25-OH₂ vitamin D₃ are reduced in patients chronically treated with antimicrobial (rifampicin) or antiepileptic (phenobarbital) drugs [28]. Both drugs activate the pregnane X receptor (PXR), a

nuclear xenobiotic receptor and ligand-activated transcription factor responsible for the inactivation and excretion of drugs. Although the levels of 1,25-OH₂ vitamin D₃ seem unaffected in PXR^{-/-} mice, these animals have low serum phosphate levels associated with reduced intestinal expression of NaPi-IIb [84]. Furthermore, activation of PXR with rifampicin was reported to induce the NaPi-IIb promoter activity.

Estrogen administration increases the Na/Pi cotransport across the intestinal epithelia by upregulating the expression of NaPi-IIb [149]. Estrogens are known to regulate the production of 1,25-OH₂ vitamin D₃ [7]. However their effect on the intestinal cotransporter is, at least partially, independent of 1,25-OH₂ vitamin D₃ as the activity of the human NaPi-IIb promoter measured in cell culture is directly stimulated by β -estradiol [149].

Normalization of the acid-base status upon metabolic acidosis involves an increased urinary excretion of protons. Acidosis leads to the release of phosphate from bones as well as to inhibition of renal reabsorption of phosphate, the later contributing to the supply of titrable acids in the urine [2,106]. In contrast, both the intestinal Na/Pi cotransport and the expression of NaPi-IIb are increased in metabolic acidosis [130], suggesting that the gut compensates for the urinary loss of phosphate.

Inherited disorders of phosphate transport in the SLC34 family

Mutations in SLC34A1 and renal phosphate handling

The role of mutations and gene variants in the SLC34A1 gene on renal phosphate handling and kidney function are still not fully understood. Prie and colleagues reported on three patients with reduced renal threshold for phosphate reabsorption ($TmP/GFR < 0.7$ mM), hypercalciuria, and kidney stones. In one patient a A48F mutation was detected, in the two other related patients a V147M mutation was found. In all three patients only one allele was found mutated and no further data were available whether there was cosegregation of the mutation with the phenotype in relatives of the patients [113]. Subsequent functional analysis of the mutations did not reveal any differences in the functional transport properties of the mutants or their trafficking in a mammalian renal cell line [140]. A second report presented patients

from a consanguinous Arab Israeli family that had rickets, hypophosphatemic Fanconi syndrome and hypercalcuria with highly elevated 1,25-OH₂ vitamin D₃ levels. The patients had all a homozygous in-frame duplication (21 bp) G154-V160dup that completely abolished phosphate induced currents in the mutant expressed in *Xenopus laevis* oocytes [94]. Since the inserted stretch of amino acids affects a putative transmembrane domain of NaPi-IIa it is very likely that the overall structure of the transporter is severely altered. The occurrence of the Fanconi syndrome is not complete since plasma bicarbonate levels are unchanged and patients are able to increase levels of active 1,25-OH₂ vitamin D₃ [94]. Moreover, in two different mouse models of *Slc34a1* deficiency either due to genetic deletion or to the spontaneous occurrence of the compound heterozygous mutations at L499V and V528M no Fanconi syndrome has been observed [15,70]. Thus, our understanding of the importance of SLC34A1 for renal phosphate handling in man and the impact of mutations on generalized proximal tubule functions is incomplete to date.

Mutations in SLC34A2: pulmonary microlithiasis

Mutations in SLC34A2 cause pulmonary microlithiasis that is in some cases associated with testicular microlithiasis [33]. Different mutations have been described including missense mutations and truncations [40]. The cellular mechanisms how mutations in SLC34A2 lead to impaired NaPi-IIb function has not been addressed to date. Pulmonary alveolar microlithiasis (PAM) is a very rare lung disease characterized by calcifications within the alveoli that may eventually lead to loss of lung function [40]. The pathogenesis of the disease has not been clarified and may relate to functions of NaPi-IIb in alveolar type II cells [133]. Whether NaPi-IIb is relevant for removing phosphate from the alveolar fluid or has other functions has not been determined. Unfortunately, no information is available whether patients with SLC34A2 mutations show other systemic disturbances of phosphate homeostasis and whether intestinal phosphate absorption is altered.

Mice lacking NaPi-IIb (*Slc34a2*) are not viable whereas inducible NaPi-IIb KO mice with deletion of *Slc34a2* in adult mice causes pulmonary calcification and reduced intestinal phosphate absorption [118]. Loss of NaPi-IIb is associated with abolished sodium-dependent phosphate transport in ileum suggesting that NaPi-IIb is the major if not only important phosphate transporter in this segment. However,

overall intestinal phosphate absorption was only mildly reduced suggesting that other segments of the small and large intestine absorb the largest quantity of phosphate and/or that other pathways such as the paracellular route may provide for major phosphate fluxes in intestine.

Mutations in SLC34A3: Hereditary hypophosphatemic rickets with hypercalcuria (HHRH)

HHRH is a rare autosomal recessive disorder caused by mutations in the SLC34A3 gene encoding for NaPi-IIc [92,19]. Patients suffer from hypophosphatemia due to renal phosphate losses. Reduced phosphate availability impairs bone mineralization and growth leading to rickets. Compensatory increases in 1,25-OH₂ vitamin D₃ synthesis and levels stimulate intestinal phosphate and calcium absorption. However, excessive calcium uptake is counterbalanced by increased urinary excretion where high urinary levels of calcium and phosphate cause nephrolithiasis and nephrocalcinosis.

Patients with HHRH showed SLC34A3 deletions and truncations but also splice site mutations, homozygous missense or compound missense mutations [18]. Missense mutations affect trafficking to the plasma membrane and/or abundance [73].

Mice lacking NaPi-IIc have been generated and surprisingly no major phenotype could be detected. Renal phosphate handling is similar to wildtype mice and no hypophosphatemia or hyperphosphaturia develops. However, NaPi-IIc show hypercalciuria and slightly increased 1,25-OH₂ vitamin D₃ levels [125]. Also a kidney-specific NaPi-IIc deficient mouse model has been developed and this mouse model has normal renal phosphate and calcium handling with no signs for altered 1,25-OH₂ vitamin D₃ metabolism (Myakala, Motta, Murer, Wagner, Koesters, Biber, Hernando, unpublished results). Thus, while NaPi-IIc has a major role in phosphate metabolism in humans, the role in mice appears to be minor.

Summary and future perspectives

Twenty years after the molecular identification of the first member of the SLC34 family, much progress has been made in understanding the hormonal regulation of these transporters, identification of various interacting proteins, and gaining insights into the transport mechanism of these transporters. Genetic studies demonstrated that all three transporters have important physiological functions. However, major questions have remained unanswered or are only partially understood. At least three fields are certainly of major interest and not fully explored to date.

Structural models of SLC34 transporters rely on structure-functions studies mostly using mutagenesis, chemical labeling and electrophysiology experiments. Obviously, crystal structures of mammalian SLC34 transporters or homologs would greatly enhance our understanding of the transport mechanism and would possibly facilitate development of isoform specific inhibitors.

NaPi-IIa served as a model protein to examine regulation of phosphate and other epithelial transporters. The identification of interacting proteins helped to understand the role of some of these proteins in epithelial physiology as exemplified for NHERF1. Nevertheless, our understanding of the "life cycle" of these transport proteins is incomplete. We miss insight into the processes regulating translation and trafficking of transporters to the cell membrane and mechanisms that affect the residence at the plasma membrane.

A third field that will require further attention is the regulation of these transporters by phosphate. This is also of major clinical importance as dysregulation of phosphate handling by intestine, bone, and kidney may contribute to chronic kidney disease, bone disease, and subsequently may increase the risk for cardiovascular diseases. FGF23 has gained much attention over the last years but the mechanisms how increases in phosphate intake or body content trigger changes in major phosphate regulating hormones such as parathyroid hormone, FGF23 or 1,25-OH₂ vitamin D₃ are largely unknown. The possibility that phosphate may regulate these transporters also independent from known factors remains also open. Thus, "phosphate-sensing" mechanisms and mediators of regulation are not fully explored and understood.

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FIGURES

Figure 1. Localization of renal phosphate cotransporters in rodent kidney

In kidney, at least three Na^+ -dependent phosphate cotransporters are involved in phosphate reabsorption: the two SLC34 family members NaPi-IIa and NaPi-IIc and the SLC20 family member Pit-2. The upper panels shows localization of the three transporters in the murine kidney cortex of animals kept on a low phosphate diet. The nephron models depict localization of the three transporter under conditions of normal-high phosphate intake (red shaded parts) which is restricted to the initial S1 segment of proximal tubules of juxtamedullary nephrons. During phosphate depletion, expression of all three transporters spreads to proximal tubules of superficial nephrons and NaPi-IIa expression extends also to S2 and S3 segments of the proximal tubule (yellow shaded nephron segments).

Figure 2. Transport mechanism of SLC34 proteins

For all SLC34 proteins, transmembrane transport for an inwardly directed Na^+ gradient, comprises an ordered sequence of binding steps in which 2 Na^+ ions in the extracellular medium bind sequentially, followed by divalent P_i and a third Na^+ ion. Cartoons indicate two conformations of the protein. Substrate release to the cytosol occurs after reorientation of the fully loaded carrier. For electrogenic NaPi-IIa and

NaPi-IIb (*top*), an intrinsic negative charge (hypothesized to be a conserved aspartic acid located between the 3rd and 4th predicted transmembrane spanning regions (Fig. 3) and which senses the transmembrane field), confers voltage dependence to transport cycle. This allows binding of the 1st Na⁺ ion (1) within the transmembrane electric field and subsequent translocation together with the other substrates (2,3,4), giving a 3:1 Na⁺:P_i stoichiometry. When substrates are released to the cytosol, the intrinsic charge senses the transmembrane field, which leads to a voltage-dependent reorientation of the empty carrier, ready for the next transport cycle. The electroneutral NaPi-IIc (*bottom*), which conserved glycine instead of aspartic acid at the critical site, has one Na⁺ ion bound (1) (like NaPi-IIa/b), but cannot be translocated [53], thus resulting in a 2:1 Na⁺:P_i transport stoichiometry. In the absence of phosphate, NaPi-IIa/b show a Na⁺-dependent leak mode, hypothesized to involve the translocation of the 1st Na⁺ ion [3]. Cartoons modified from [47].

Figure 3. Topology and structure-function features of SLC 34 proteins

Topology model for SLC34 show the predicted transmembrane domains (*numbered*) and repeat regions (*dark shading*) for each family. The 3-D folding is currently unknown. For SLC34 evidence suggests that the reentrant regions (3,4) and (8,9) physically associate to form a substrate coordination site [52]. The disulfide bridge and two glycosylation sites in the large extracellular loop are indicated for the SLC34 proteins. Critical residues for targeting/regulation and electrogenicity are highlighted.

Figure 4. Network of proteins interacting with NaPi-IIa in renal proximal tubule cells

NaPi-IIa interacts with the four members of the NHERF family as well as with Shank in the brush border membrane. These interactions take place between the C-terminal PDZ-binding motif of NaPi-IIa (TRL) and the PDZ domains of the NHERFs/Shank proteins indicated with an asterisk. Association with NHERF1/2 controls the stability of the cotransporter in the apical membrane. In addition, NHERF1/2 may play a role in the PTH-induced downregulation of NaPi-IIa by determining the intracellular

signaling activated upon receptor stimulation. The physiological meaning of the association of NaPi-IIa with NHERF3/4 is less clear and it is not discussed in this review. In addition to a PDZ-domain, Shank2E also contains ankyrin repeats as well as SH3 and prolin-rich domains that are not shown in the Figure. PTH-R: PTH receptor; PLC: phospholipase C; PDZ: post-synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1) and Zonula occludens (ZO-1). MERM-BD: merlin-ezrin-radixin-moesin-binding domain.

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Figure 1

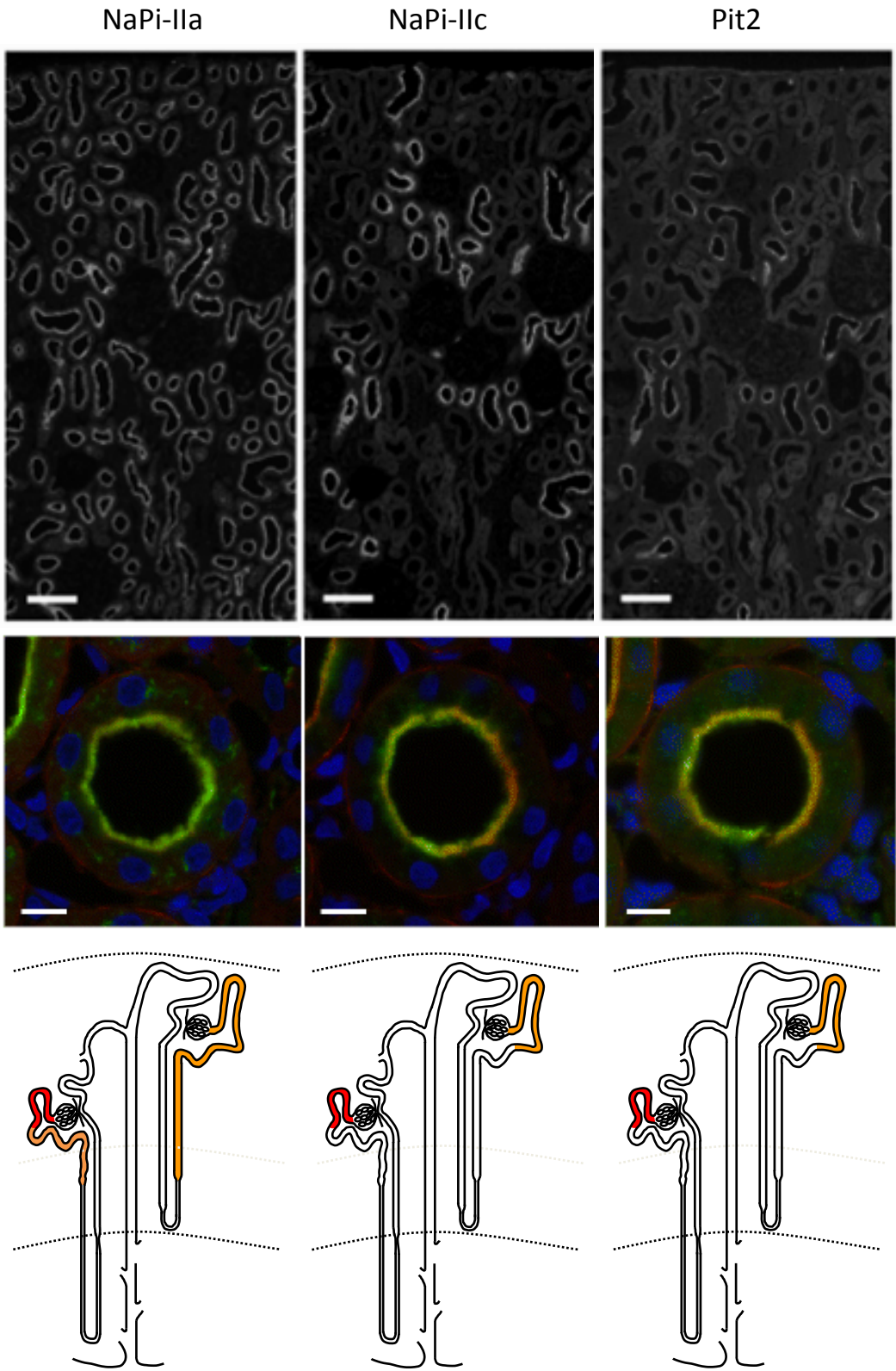


Figure 2

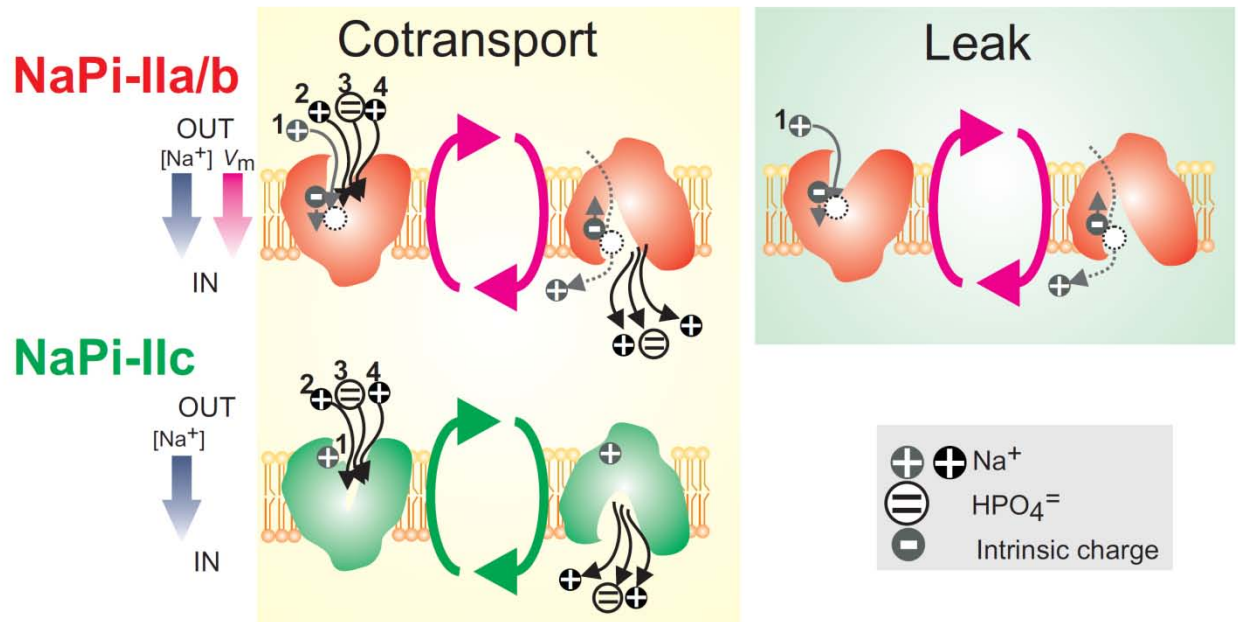


Figure 3

